AD_____

Award Number: W81XWH-05-1-0566

TITLE: Targeting Mechanisms of Resistance to Taxane-Based Chemotherapy

PRINCIPAL INVESTIGATOR: Chung-Ying Huang, M.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, WA 98109

REPORT DATE: September 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2007	Annual Summary	1 SEP 2006 - 31 AUG 2007

4. TITLE AND SUBTITLE

Targeting Mechanisms of Resistance to Taxane-Based Chemotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0566

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Chung-Ying Huang, M.D.

E-Mail: cyhuang@u.washington.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fred Hutchinson Cancer Research Center
Seattle, WA 98109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Neoadjuvant chemotherapy, microarray

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	17	**19b. TELEPHONE NUMBER** *(include area code)*

Patients with high-risk localized prostate cancer have a high recurrence rate following primary therapy. Neoadjuvant chemotherapy has been shown to be beneficial in reducing recurrence rates in some tumor types, but has yet to be of proven benefit in prostate cancer. Further, current clinical, pathological and molecular markers poorly predict the response and resistance of chemotherapy, and the molecular mechanisms of chemotherapy resistance are largely unknown. We utilized tissue resources from a unique prospective phase II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone in patients with high-risk localized prostate cancer to identify molecular alterations after chemotherapy, and correlated these alterations with clinical and pathological indicators of tumor response. We hypothesized that this approach may identify molecular signatures of chemotherapy resistance and uncover mechanisms or pathways suitable for targeting with the objective of improving tumor responses to chemotherapy.
Gene expression changes after chemotherapy were measured in 31 patients who completed 4 cycles of docetaxel and mitoxantrone neoadjuvant chemotherapy. The chemotherapy induced profile was further correlated with clinical outcome including percentage of PSA decline and PSA-relapse free survival. Cytokines and cytokine pathways were found to be associated with immediate clinical outcome, measured by percentage of PSA decline. Four cytokines including IL8, CXCL10, IL1B and CCL2 were included for further analysis. Expression changes of these 4 cytokines by qRT-PCR were correlated with percentage of PSA decline. Expression changes of IL8 and CXCL10 were significantly and negatively associated with percentage of PSA decline. Further in vitrotests showed only CXCL10 but not IL8 conferring chemoresistance to prostate cancer cells. When using longer term clinical outcome, we found genes correlated with PSA-relapse free survival. Of these, patients with PSA relapse tended to have higher MAOA expression after chemotherapy. In vitro cell culture test further confirmed adding MAOA inhibitor may have additive effects to docetaxel to inhibit prostate cancer cell growth.
In summary, correlations between chemotherapy induced profiles and clinical outcomes including percentage of PSA decline and PSA-relapse free survival identified candidate genes and pathways that may contribute to chemotherapy resistance and response. Cytokines were recognized as having important effect to modify chemoresistance of prostate cancer. In addition, MAOA may have a longer term effect on chemoresistance of prostate cancer. Combining these results, development of small molecules or monoclonal antibody to modify cytokine expression and utilizing existing drugs such as MAOA inhibitors may modify chemotherapy response in patients with prostate cancer. Further in vivotests to confirm the above findings are needed.

Table of Contents

INTRODUCTION:

Chemotherapy with docetaxel and/or mitoxantrone has been shown to be beneficial for some patients with advanced hormone refractory prostate cancer [1, 2]. However, there are no useful clinical and pathological markers to predict who will benefit from receiving these agents. In addition, the mechanisms used by tumor cells to circumvent the cytotoxic effects of chemotherapy are poorly understood, and thus cannot be effectively targeted to enhance tumor responses. Our hypothesis is that identifying *in vivo* gene expression changes before and after neoadjuvant chemotherapy will uncover the molecular mechanisms of prostate tumor response and resistance to cytotoxic drugs. Once identified, these tumor resistance mechanisms can be exploited through the design of combination therapies targeted toward inhibiting resistance pathways.

BODY:

Between January 2001 and November 2004, 57 patients with high-risk localized prostate cancer (defined as TNM > cT2b or T3a or PSA $\geq$ 15 ng/ml or Gleason grade $\geq$ 4+3) were recruited for a phase II trial clinical trial of neoadjuvant chemotherapy. The design of the clinical trial has been previously described [3, 4]. Figure 1 shows the schema of the study design. From each patient, ten standard prostate biopsies (bilateral at the apex, bilateral medial and lateral at mid-gland, bilateral medial and lateral at the base of the gland) were obtained under ultrasound guidance and snap-frozen in liquid nitrogen prior to chemotherapy. At the time of radical prostatectomy, cancer-containing tissue samples were snap frozen immediately after prostate removal. Evaluation of tissue samples identified the presence of adequate numbers of cancer cells in both pre-treatment and post-treatment samples for 31 subjects. We used laser capture microdissection techniques to specifically collect cancer epithelia from pre-treated biopsy specimens and post-treated radical prostatectomy specimens. Total RNA and cy3-cy5 labeled cDNA were generated based on the standard protocol in our lab. The strategy of hybridization is depicted in Figure 1.

Expression Profiles Reflect Differential Prostate Cancer Responses to Chemotherapy

We have previously reported the generation of a chemotherapy induced gene expression profile. We also validated one candidate chemotherapy resistance gene, GDF15, having cytoprotective effect toward prostate cancer cells against docetaxel and mitoxantrone. In our 2006 report, we delineated a general mechanism of chemotherapy resistance by comparing the gene expression profiles between pre-treated and post-treated samples. To investigate individual susceptibility to chemotherapy resistance and response, we need to correlate expression profiles with clinical outcomes such as PSA change after chemotherapy, PSA-relapse free survival, or pathological change after chemotherapy. However, detailed histopathological reviews of all radical prostatectomy samples in this study did not reveal any patient with a complete response, and partial pathological responses are difficult to accurately quantify, as the pre-treatment assessment of tumor volume is based on sampling by needle biopsies rather than having a complete organ for comparison. Thus, we used percentage of PSA decline after chemotherapy as an immediate clinical endpoint to explore possible association between chemotherapy induced profile and clinical outcome. PSA response is an immediate endpoint calculated from the serum PSA level measured before, during, and after chemotherapy. We have previously reported that the serum androgen levels were not affected by the chemotherapy protocol employed here [4]. Thus PSA concentrations likely reflect changes in cancer cell numbers, though could potentially represent chemotherapy-mediated changes in cellular secretory mechanisms or tumor vasculature. Using SAM analysis (Significant Analysis of Microarray, http://www-stat.stanford.edu/~tibs/SAM/), we incorporated chemotherapy-induced gene expression profile and percentage of PSA decline after chemotherapy into a regression model. Percentage of PSA decline was defined as the maximal percentage decline of PSA level after chemotherapy compared with baseline PSA level before chemotherapy. The majority of patients had

PSA declines after chemotherapy, however; six out of 31 patients had PSA elevations after chemotherapy (Figure 2). This might suggest inherent chemotherapy resistance among these 6 patients. SAM regression analysis showed that 26 upregulated genes associated with poorer chemotherapy response (FDR less than 25%). Only one downregulated gene associated with poorer chemotherapy response (Figure 2). Of these 27 genes, several assocated with particular functional roles including: MAPK pathway (Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A and MAPK6), anti-apoptosis gene (Baculoviral IAP repeat-containing 2), transporter (Potassium voltage-gated channel subfamily H member 8, Solute carrier family 22 member 3) and the cytoskeleton. Gene pathway analysis showed 131 gene sets that associated with chemotherapy responses based on percentage of PSA decline. The top 10 gene sets included immune response (chemokine activity and receptor, JAK-STAT cascade, and response to bacterium), cell signaling (G-protein-coupled receptor binding and G-protein signaling coupled to cyclic nucleotide second messenger), musculoskeletal-associated genes (myosin light chain kinase activity and muscle development) and anti-apoptosis genes. As we have reported previously, several chemokines were not only induced by chemotherapy but were also shown to be associated with individual susceptibility to chemotherapy response (e.g. IL8). Expression of IL8 was significantly induced after chemotherapy. Furthermore, higher expression of IL8 associated with poorer chemotherapy response based on SAM regression analysis (Figure 2). We next measured gene expression changes among several chemokines including IL8, CCL2, CXCL10 and IL1B by quantitative real-time PCR (qRT-PCR). Expression changes measured by qRT-PCR were correlated with percentage of PSA decline by a univariate liner regression model. We found only IL8 and CXCL10 were significantly associated with percentage of PSA decline (Table 2 and Figure 3). Patients with higher expression of IL8 or CXCL10 seemed to be more resistant to chemotherapy with less percentage of PSA decline after chemotherapy.

CXCL10 but not IL8 may influence chemotherapy resistance

We further investigated the paracrine effect of CXCL10 and IL8 on prostate cancer cells. The LNCAP cell line was chosen for further study because the baseline expression levels of these chemokines were extremely low by quantitative real-time PCR and Western blot. LNCAP cells were treated with various concentration of recombinant CXCL10 and IL8 (R&D Systems). CXCL10 alone inhibited the proliferation of LNCAP cells, consistent with previous report [5]. However, increased concentrations of CXCL10 increased the proportion of viable cells when also treated with docetaxel and mitoxantrone (Figure 3). Thus, CXCL10 may not only inhibit cancer cell proliferation but also confer cancer cell resistance to chemotherapy. On the other hand, although IL8 was significantly correlated with percentage of PSA decline; MTS assay of LNCAP cells treated by recombinant IL8 did not show any modifying effect toward chemoresistance (Figure 3). IL8 has been suggested to have no effect in modifying chemoresistance in an osteosarcoma cell line [6]. Our finding of IL8 in prostate cancer cell is in agreement with the previous report.

Expression change of MAO-A may influence PSA free relapse

Percentage of PSA decline is an immediate clinical outcome, which may not completely reflect the longer-term prognosis of prostate cancer treated by chemotherapy. We further analyzed the chemotherapy induced profile against a third endpoint involving the determination of PSA-relapse free survival. For patients treated by radical prostatectomy, PSA serum levels are a good indicator of persistent or recurrent tumor when a threshold of 0.4 ng/ml and rising is used as an indicator of ultimate progression to metastasis [4]. We defined PSA relapse as a patient having two consecutive PSA elevations greater than 0.4 ng/ml. There are 11 out of 31 patients having PSA relapse to date. A survival analysis method (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used to profile differentially expressed genes associated with PSA-relapse free survival. Using default p-value of 0.001, we found 9

significant genes that were associated with PSA relapse-free survival including microtubule associated genes - tubulin-specific chaperone d and clathrin heavy polypeptide; membrane associate protein - metadeherin, transmembrane trafficking protein and pro-oncosis receptor inducing membrane injury gene and nardilysin; and three hypothetical proteins. Docetaxel directly acts on microtubule assembly and disassembly; and thus it is not surprising to see two microtubule associated genes associated with outcomes. In order to explore more candidate PSA relapse-free survival associated genes, we loosened the p-value to 0.01 and found 141 survival associated genes. Of the 141 genes, 101 were up-regulated and 40 genes were down-regulated in patients with PSA relapse. One of the survival associated genes; topoisomerase II alpha, was downregulated in patients with PSA relapse (Figure 5A), which is consistent with previous study showing chemosensitive testis cell lines had higher expression of TOP2A [7]. Another interesting gene; monoamine oxidase A (MAOA) was upregulated in patients with PSA relapse (Figure 5A). Quantitative real-time PCR (qRT-PCR) was performed to validate the MAOA expression change after chemotherapy. The expression change of MAOA by qRT-PCR was incorporated into a Cox Proportional Hazard Model using time to PSA relapse as the clinical outcome. Expression change of MAOA alone was statistically associated with PSA relapse-free survival by Cox model (hazardous ratio = 1.66, p-value= 0.027). After adjusting by Gleason sum score, expression change of MAOA still was marginally significant (hazardous ratio = 1.55, p-value= 0.068), which suggests that MAOA is an independent survival risk factor. We noticed that expression change of MAOA after chemotherapy was upregulated in patients with PSA relapse compared with patients without PSA relapse. We further investigated whether expression of MAOA may influence chemoresistance. We first tested whether chemotherapy exposure induces MAOA activity. Using MAO-Glo assay system (Promega), MAO activity was induced by docetaxel in LNCAP cells (Figure 5B). In order to see whether inhibition of MAOA activity will modify chemoresistance of prostate cancer cells, we treated LNCAP cell first with MAOA inhibitor (Clorgyline, Sigma) and then added docetaxel one hour after MAOA inhibitor administration. MTS cell proliferation assay was performed at 24 hours and 48 hours after docetaxel treatment. We found at 24 hours, MAOA inhibitor seemed to have an addictive effect (although not statistically significant) to low concentrations of docetaxel (10^{-9} and 10^{-8}M). The addictive effect was not as substantial at higher concentration of docetaxel (10^{-7}M, Figure 5C). At 48 hours, we still observed an addictive cell-inhibitory effect of MAOA inhibitor to low concentration docetaxel (Figure 5D). However, we did not observe any effect with mitoxantrone. The above results suggest MAOA expression may specifically influence chemoresistance of prostate cancer cells to docetaxel.

MAOA oxidizes neurotransmitters and dietary amines and influences the process of neurotransmitter metabolism as well as cell growth and differentiation. The byproduct of MAOA, aminoaldyhyde and hydrogen peroxide, may influence cell growth and differentiation [11]. Our previous study has shown prostate cancers with higher Gleason grade have higher MAOA protein expression [12]. Hence, MAOA may be related to tumorigenesis and/or tumor progression, but its roles in stress response and cell death are largely unknown. MAOA inhibitors may protect neuronal cells from apoptosis [8, 9]. Another study showed MAOA-inhibitors increased survival of non-tumorigenic keratinocyte treated by irradiation and cisplatin; however, MAOA inhibitor did not affect survival of HPV-16 transfected keratinocyte and PC3 cells and even decreased survival of a turmorigenic cell, ras-transfected keratinocyte [10], suggesting the effect of MAOA inhibitor on cell survival may be different between different tissue types and/or between normal and tumor cells. Our array findings suggest overexpression of MAOA after chemotherapy may confer resistance to chemotherapy and *in vitro* tests suggest inhibition of MAOA activity concurrently with docetaxel may further enhance cytotoxic effects.

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified alterations of gene expression associated with percentage of PSA decline after chemotherapy. Of the changes observed, the elevated expressions of several chemokines were specifcally associated with PSA decline.
- We have found that alterations of CXCL10 but not IL8 protein level may influence chemoresistance of prostate cancer cells.
- Preliminary analyses involving PSA-relapse free survival revealed genes that were associated with PSA-relapse free survival. Of these genes, we found patients with PSA relapse had higher expression of MAOA based on microarray and qRT-PCR analysis. *In vitro* cell culture systems suggested that inhibition of MAOA activity may have additive effects with docetaxel to inhibit prostate cancer cell growth.

REPORTABLE OUTCOMES:

Chung-Ying Huang, TomaszM. Beer, Celestia S. Higano, Lawrence D. True, Robert Vessella,Paul H. Lange, Mark Garzotto, and Peter S. Nelson. MolecularAlterations in Prostate Carcinomas that Associate with *In vivo* Exposure to Chemotherapy: Identification of a Cytoprotective Mechanism Involving Growth Differentiation Factor 15. *Clin Cancer Res* 2007;13(19), 2007 (in press).

CONCLUSION:

We have identified differentially expressed genes in post-treated prostate tumor tissue associated with immediate clinical outcomes of percentage of PSA decline and longer-term outcomes of PSA relapse-free survival. Combining our studies from the previous year, we found that cytokines may play important roles in modifying the chemoresistance of prostate cancer. We also showed CXCL10 but not IL8 may contribute to the chemoresistance to prostate cancer cells. In addition, expression changes of MAOA after chemotherapy may be related to PSA relapse-free survival. A cell proliferation assay showed MAOA inhibitor exerts addictive effects to docetaxel on prostate cancer cells. Improvement of chemotherapy responsiveness may be feasible by manipulating the expression level of cytokines or adding inhibitor molecules targeting genes/proteins altered in response to treatment. Further studies and *in vivo* assays to confirm the clinical relevance of the above findings are needed in the future.

REFERENCES:

1. Tannock, I.F., et al., *Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer.* N Engl J Med, 2004. **351**(15): p. 1502-12.
2. Petrylak, D.P., et al., *Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer.* N Engl J Med, 2004. **351**(15): p. 1513-20.
3. Beer, T.M., et al., *Phase I study of weekly mitoxantrone and docetaxel before prostatectomy in patients with high-risk localized prostate cancer.* Clin Cancer Res, 2004. **10**(4): p. 1306-11.
4. Garzotto, M., et al., *Neoadjuvant mitoxantrone and docetaxel for high-risk localized prostate cancer.* Urol Oncol, 2006. **24**(3): p. 254-9.
5. Nagpal, M.L., J. Davis, and T. Lin, *Overexpression of CXCL10 in human prostate LNCaP cells activates its receptor (CXCR3) expression and inhibits cell proliferation.* Biochim Biophys Acta, 2006. **1762**(9): p. 811-8.
6. Duan, Z., et al., *Overexpression of IL-6 but not IL-8 increases paclitaxel resistance of U-2OS human osteosarcoma cells.* Cytokine, 2002. **17**(5): p. 234-42.
7. Fry, A.M., et al., *Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines.* Cancer Res, 1991. **51**(24): p. 6592-5.
8. Ou, X.M., K. Chen, and J.C. Shih, *Monoamine oxidase A and repressor R1 are involved in apoptotic signaling pathway.* Proc Natl Acad Sci U S A, 2006. **103**(29): p. 10923-8.

9. Yi, H., et al., *Type A monoamine oxidase is the target of an endogenous dopaminergic neurotoxin, N-methyl(R)salsolinol, leading to apoptosis in SH-SY5Y cells.* J Neurochem, 2006. **96**(2): p. 541-9.
10. Seymour, C.B., et al., *Monoamine oxidase inhibitors l-deprenyl and clorgyline protect nonmalignant human cells from ionising radiation and chemotherapy toxicity.* Br J Cancer, 2003. **89**(10): p. 1979-86.
11. Pietrangeli, P. and B. Mondovi, *Amine oxidases and tumors.* Neurotoxicology, 2004. **25**(1-2): p. 317-24.
12. True, L., et al., *A molecular correlate to the Gleason grading system for prostate adenocarcinoma.* Proc Natl Acad Sci U S A, 2006. **103**(29): p. 10991-6.

APPENDICES:

Table 1. Significant GO functional categories affecting percentage of PSA decline

GO category	GO term	GO description	LS Permutation p-value	KS Permutation p-value
1664	MF	G-protein-coupled receptor binding	1.00E-05	0.004
4687	MF	myosin light chain kinase activity	1.00E-05	0.001
8009	MF	chemokine activity	1.00E-05	0.004
42379	MF	chemokine receptor binding	1.00E-05	0.004
6874	BP	calcium ion homeostasis	1.00E-05	0.000
6916	BP	anti-apoptosis	1.00E-05	0.002
7187	BP	G-protein signaling\, coupled to cyclic nucleotide second messenger	1.00E-05	1.00E-05
7259	BP	JAK-STAT cascade	1.00E-05	0.000
7517	BP	muscle development	1.00E-05	1.00E-05
9618	BP	response to bacterium	1.00E-05	1.00E-05

Table 2. Univariate linear regression analysis of expression changes of chemokine and percentage of PSA decline.

Chemokines	Regression Coefficient	P-value	95% CI
IL8	**-3.01**	**0.02**	**-5.55 ~ -0.47**
IL1B	-1.20	0.52	-4.94 ~ 2.55
CXCL10	**-5.86**	**0.02**	**-10.70 ~ -1.03**
CCL2	1.26	0.49	-2.40 ~ 4.93

Figure 1. Study design and hybridization strategy

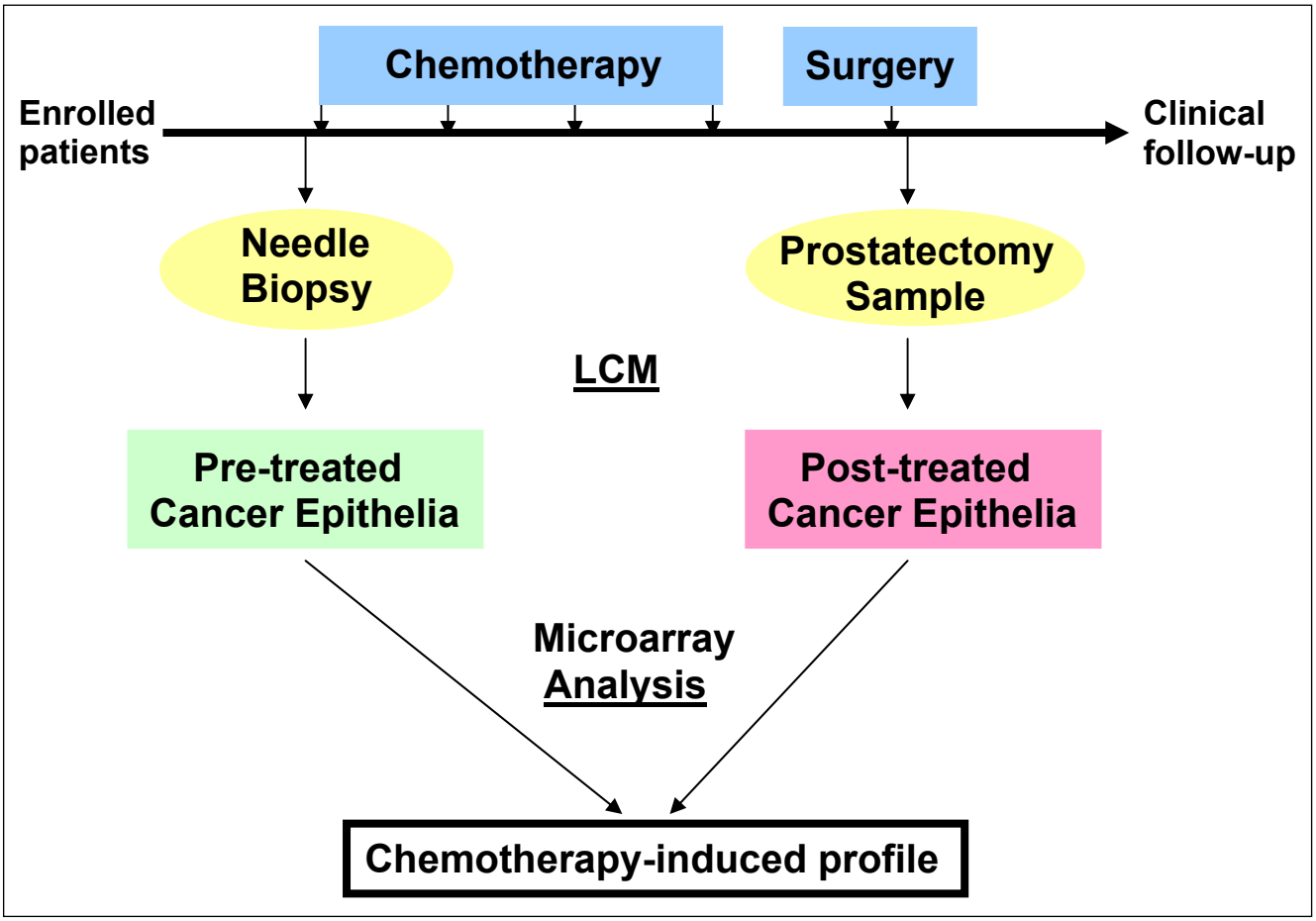


Figure 2. Differentially expressed genes associated with percentage of PSA decline after chemotherapy. (FDR < 25%)

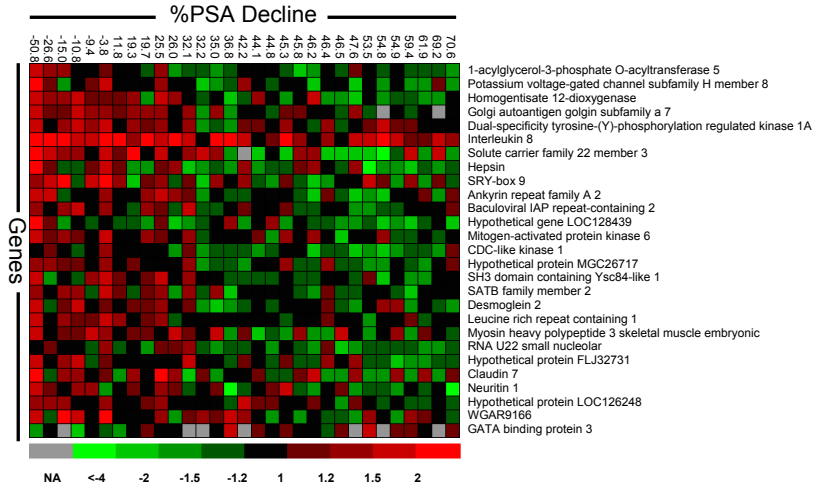
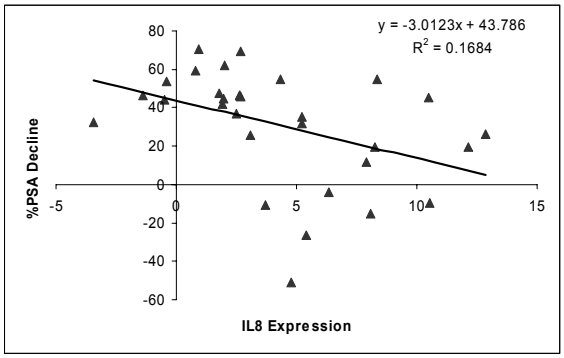
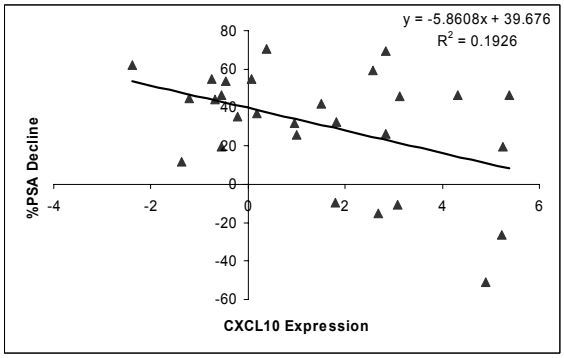
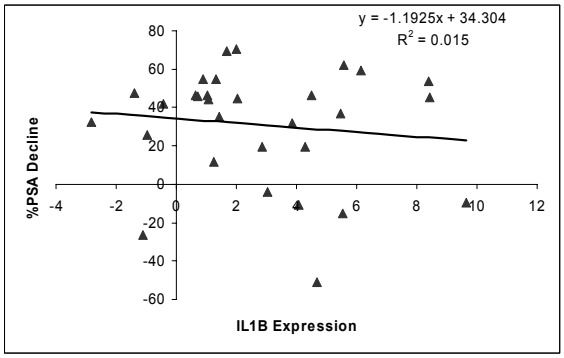


Figure 3. Linear regression of %PSA decline and expression changes of chemokines after chemotherapy. (X-axis: Cycle threshold difference between pre-treatment and post-treatment samples; Y-axis: percentage of PSA decline)







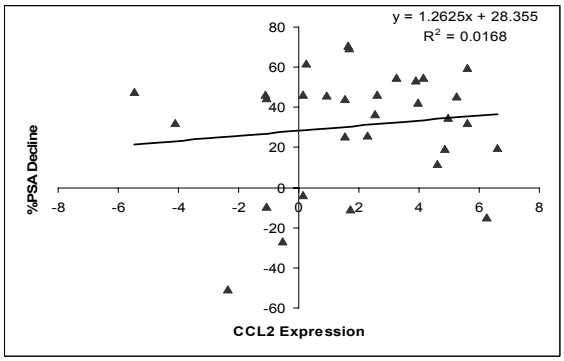


Figure 4. MTS assay of cell lines treated with a concentration series of recombinant IL8 and CXCL10 protein. Viability percentage in the treatment group was calculated by dividing OD490 values of chemokine-treated cell groups by no-chemokine treated control group. Percentage of viability in docetaxel and mitoxantrone treatment groups was calculated by dividing OD490 value of docetaxel or mitoxantrone treated cells by the no treatment cells at corresponding concentrations of chemokines. * p-values < 0.05 by student *t*-test.

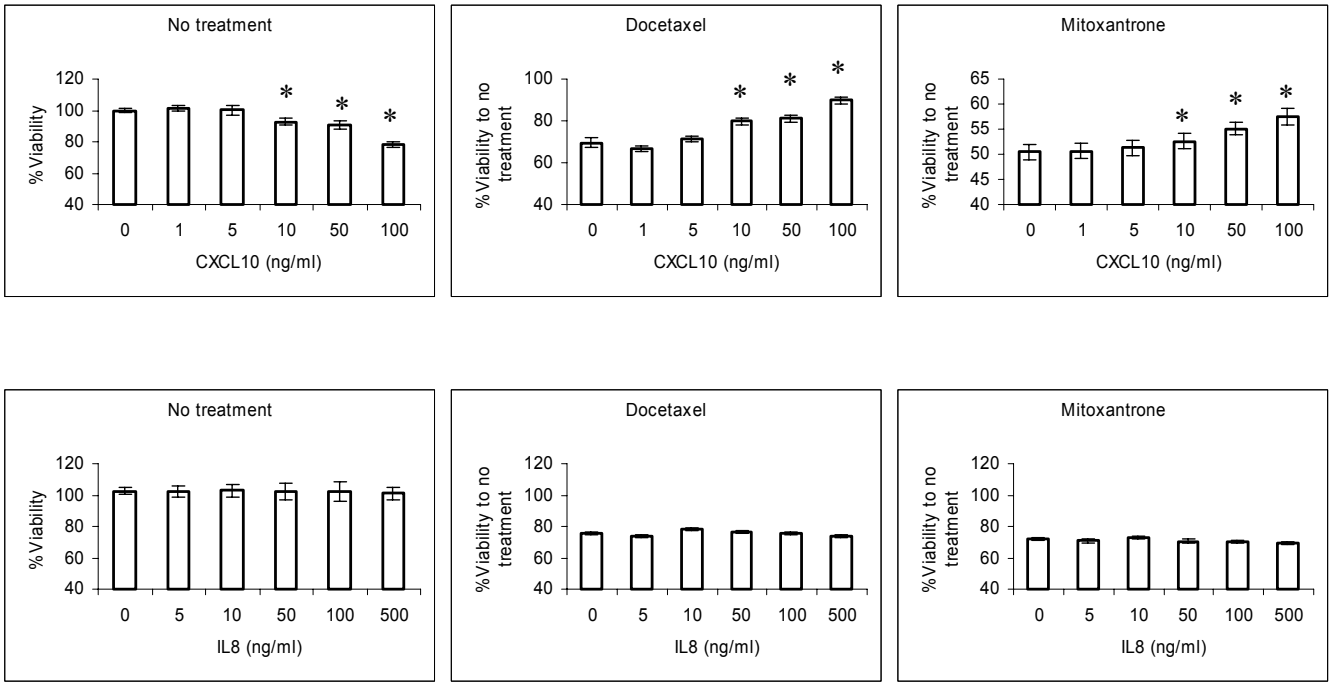
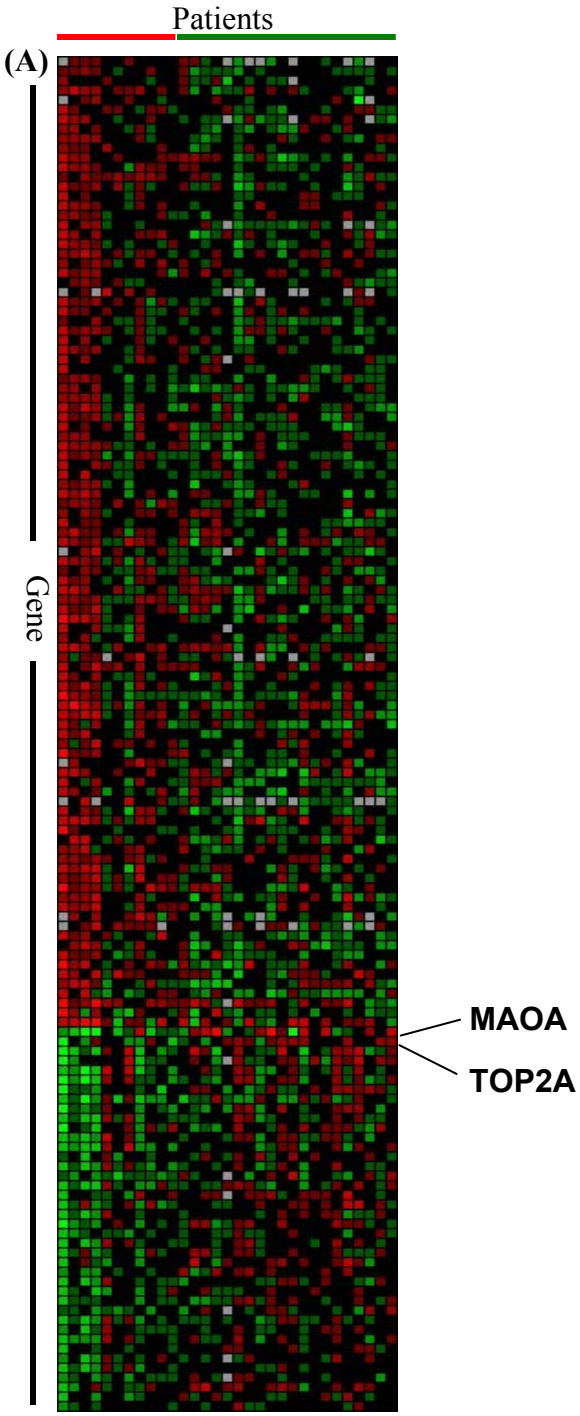
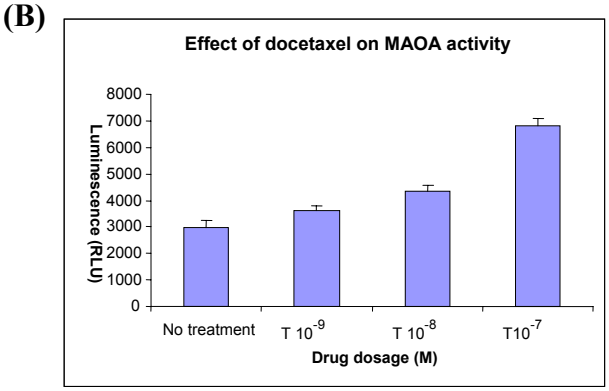
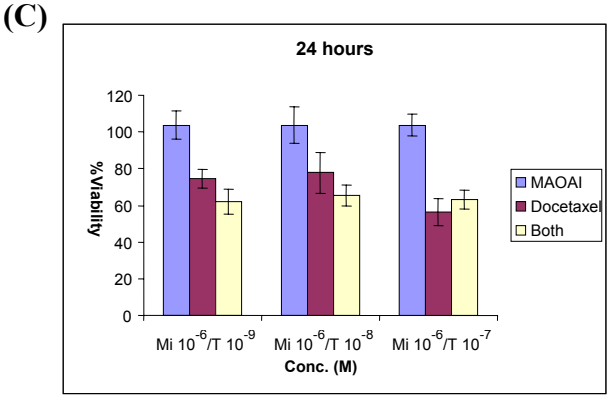
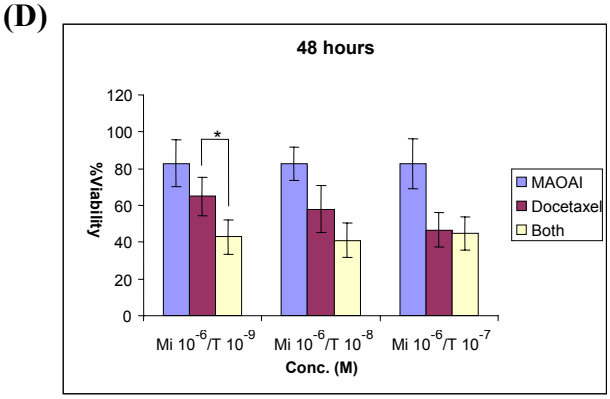


Figure 5. (A) Differentially expressed genes by survival data analysis of chemotherapy induced profile correlated with time to PSA relapse. Patients highlighted by red bar had PSA relapse and patients highlighted by green bar had no PSA relapse to date. (B) MAOA activity is induced by increasing concentrations of docetaxel. MTS cell proliferation assay of LNCAP cell. (C) 24 and (D) 48 hours after treatment with docetaxel and MAOA inhibitor. MAOA inhibitor was given one hour before administration of docetaxel into cell culture medium. %Viability was the proportion of viable cells to no treatment control cells. The concentration of MAOA inhibitor was constant through different experiments at 10^{-6}M. The concentration of docetaxel ranged from 10^{-9} to 10^{-7}M. Blue bars represent MAOA inhibitor treatment alone. Purple bars represent docetaxel treatment alone. Yellow bars represent combination treatment of MAOA inhibitor and docetaxel. (Mi: MAOA inhibitor; T: docetaxel, * p-value < 0.05 by Student t-test)


Patients
(A)
Gene
MAOA
TOP2A


(B)
Effect of docetaxel on MAOA activity
Luminescence (RLU)
8000
7000
6000
5000
4000
3000
2000
1000
0
No treatment
T 10-9
T 10-8
T10-7
Drug dosage (M)


(C)
24 hours
%Viability
120
100
80
60
40
20
0
Mi 10-6/T 10-9
Mi 10-6/T 10-8
Mi 10-6/T 10-7
Conc. (M)
MAOAI
Docetaxel
Both


(D)
48 hours
%Viability
120
100
80
60
40
20
0
*
Mi 10-6/T 10-9
Mi 10-6/T 10-8
Mi 10-6/T 10-7
Conc. (M)
MAOAI
Docetaxel
Both